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13. ABSTRACT *(Maximum 200 words)*
The regulation of the CaM KI signaling pathway will be studied in human breast cancer cell lines. We hypothesize that CaM KI phosphorylation of the transcription factor, CREB is a key regulatory step in cell cycle regulation and/or prevention of apoptosis. Since tamoxifen (TAM) causes cell cycle arrest and apoptosis in breast cancer cells, we thought that part of TAM's effects may be mediated by inhibition of the CaM kinase cascade. Regardless if this is the case, CaM inhibitors cause cell cycle arrest and apoptosis in many tumor cells. Hence, CaM and its targets are potential sites of therapeutic intervention.

We have found that TAM does not inhibit CaM activation of CaM KI as originally proposed. We have found, however, that Ca^{2+} not only modulates the phosphorylation of S133 of CREB, but also CREB binding protein (CBP) transactivation. In characterizing the mechanism of CaM KI activation by CaM KKB, we found that the substrate specificity of CaM KI changes upon T177 phosphorylation by CaM KK. These data will be very relevant to how CaM KI can function in breast cancer cells, particularily if different substrates require alternate modes of enzyme activation. As cells reenter the cell cycle from growth arrest, CaM KI expression dramatically peaks. It will be interesting to see at which stages during the cell cycle CaM KI is functionally important and whether these stages are compromised in our human breast cancer cell lines.

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Introduction

Calmodulin (CaM) activates a family of ser/thr protein kinases called CaM kinases. Classically, the CaM kinases are organized with an N-terminal kinase domain, followed by an autoinhibitory domain, and a C-terminal CaM binding domain. The protein CaM has N and C-terminal lobes each of which bind 2 Ca^{2+} ions. Ca^{2+} binding exposes hydrophobic pockets on each domain that enable CaM to recognize key hydrophobic residues within the CaM-binding domains of target proteins. Ca^{2+}/CaM binds to the CaM kinase family members, removes the autoinhibitory domain from the active site and allows catalytic activity.

Despite bing the first identified CaM kinases, very little is known in regards to the regulation or biological significance of CaM KI. It has recently been appreciated that in addition to strict dependence on Ca^{2+}/CaM, CaM KI must also be phosphorylated to be maximally active (1,2). The activating phosphorylation was originally interpretted as autocatalytic based on its dependence on Ca^{2+}/CaM. However, it was simultaneously reported that similar levels of activation (20-50 fold) were acheived by an activator separated from CaM KIa during the latter's purification (3). This activator protein has since been purified from both porcine and rat brain and was made available to us.

We have isolated the human cDNA of CaM KI to study its regulation and activation. The activator is a protein kinase that phosphorylates CaM KI on the predicted site of autophosphorylation, T177 (4). This residue is at an equivalent position to the activating phosphorylation sites in cAMP-dependent protein kinase (PKA), PKC, MAP kinases ERK1 and 2, MAP kinase kinase, and cyclin dependent kinases CDC2, CDK2, and CDK4. The solution of the crystal structures of PKA (5), ERK2 (6), CDK2 (7), and most recently CaM KI (8), indicate that this residue is part of the "activation loop" at the entrance to the catalytic pocket. It is proposed that a negative charge at this position within the loop aligns the residues critical for catalysis (9). Activation loop phosphorylation and relief of intrasteric autoinhibition by Ca^{2+}/CaM represent two distinct and fundamentally independent mechanisms, both of which are essential for CaM KI to be maximally active (4). Through mutational analysis of CaM KI, we have demonstrated that Ca^{2+}/CaM plays several roles in activation. Ca^{2+}/CaM-binding to CaM KI removes the autoinhibitory domain and exposes T177 to the activator, now designated CaM kinase kinase (CaM KK). In addition, CaM KK itself is dependent on Ca^{2+}/CaM for its activity (4).

The activator preparation was fractionated into two peaks of activity each capable of phosphorylating T177 of CaM KI (10). Protein sequence analysis revealed that each peak represented a distinct protein kinase. Both kinases also phosphorylate and activate CaM KIV by phosphorylation of the equivalent thr (11). We have cloned both activators and designated them CaM KK A and B. CaM KK A is identical to the CaM KIV activator reported by Soderling's group (12). CaM KK B has not previously been reported. We have done extensive characterization of activation of CaM KI and IV by CaM KK A and B and now would like to elucidate the role of activation *in vivo*.

A number of years ago, before CaM KI was cloned, it was shown by Greenberg and colleagues to phosphorylate the cAMP-response element binding protein (CREB) on the activating residue, S133 (13). CREB family members dimerize and this phosphorylation enables the dimers to interact with its cofactor, CREB-binding protein (CBP). CaM KI and IV have very recently been shown to activate CREB-mediated transcription *in vivo* (14, 15, 16). CaM KII, however, inhibits CREB-mediated transcription by phosphorylating not only S133 but also an additional residue, S142 (14, 15).

Recently, transgenic mice have been generated which express a dominant-negative form of CREB. The dominant-negative is created by changing the activating ser to ala. This protein is capable of dimerizing but is ineffective for transcription. The studies of Barton et al. (17) indicate that T cells from these mice, display a G1 cell cycle arrest, a 90%

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decrease in viability, and a significant increase in apoptosis. Normally, stimulation of the T cell receptor (TCR) results in increases in the mRNA of several genes. This TCR-stimulation of transcription is almost completely ablated for the c-jun, c-fos, Fra-2, and FosB transcripts in the CREB dominant-negative mice. These data indicate that CREB is an essential regulator of cell proliferation and without CREB cells die from apoptosis.

Interestingly, our lab sees a similar phenotype in transgenic mice overexpressing a kinase-inactive CaM KIV in T cells. These mice have a 10-fold lower number of thymic T cells and a significant number of apoptotic cells as compared to mice overexpressing wt CaM KIV. In addition, T cells from kinase-minus mice, that are stimulated through the TCR, generate significantly lower levels of phospho-CREB as compared to controls. As a result of decreased phospho-CREB, induction of c-fos and c-jun after TCR stimulation is also dampened. Lower levels of these transcription factors results in the inability to transcribe the IL-2 gene (18). Taken together, these data suggest that CaM KIV-mediated phosphorylation of CREB is vital to TCR-stimulated proliferation and prevention of apoptosis. CaM KIV, however, has a tissue distribution limited to brain, thymus, spleen, epididymis, and testis (19, 20, 21). Since CaM KI is regulated by the same CaM KKs as CaM KIV and CaM KI also activates CREB, it is likely that in the breast cancer cell, CaM KI plays an analogous role as CaM KIV in the T cell. We will investigate CaM KI activation of CREB-mediated transcription in human breast cancer cell lines.

One of the drugs widely used in the treatment of breast cancer is Tamoxifen (TAM). The major mechanism of action of the drug is that it binds competitively to the estrogen receptor (ER), blocking the mitogenic signal which usually follows receptor activation. Thus, the drug is particularly useful in tumors which express the ER. TAM has, however, also been shown to be beneficial in patients with ER-negative tumors, as well as in cases where hormone dependence has not been established. Likewise, the drug leads to G0/G1 cell cycle arrest and subsequent apoptosis in breast cancer cells that do not express the ER. These data suggest other mechanisms of TAM action.

One such possibility stems from the observation that TAM is also antagonistic to calmodulin (CaM) function. Interestingly, there is a vast literature indicating that CaM is an essential protein for progression through the cell cycle, specifically G0/G1, G1/S, G2/M, and anaphase to metaphase (22, 23, 24, 25). In the filamentous fungus, *Aspergillus nidulans*, the CaM gene is essential and when it is not functional, cells are unable to proliferate (26). Similarly, decreasing the amount of CaM in cultured mammalian tumor cells results in cell cycle arrest while CaM inhibitors induce apoptosis. Thus, CaM-dependent pathways could represent targets for additional breast cancer therapies.

We hypothesize that CaM KI phosphorylation of CREB is a key step for cell cycle progression and prevention of apoptosis. Collectively, these data also suggest that another mechanism of TAM action may be blocking this CaM kinase signaling cascade. We propose to study the regulation of this pathway in ER-positive and ER-negative human breast cancer cells and will ascertain which components in the cascade can be inhibited by TAM or other agents. In addition, phenotypic changes due to blocking this pathway such as apoptosis, cell cycle arrest, changes in proliferation rate, or alteration of the duration of the cell cycle phases will be analyzed.

Body

Results and Discussion

In my proposal's statement of work section, tasks # 1-3 were to be completed within the first 12 months of funding. The primary part of this discussion will be to address these specific aims. As preliminary studies for task #2 which is "determine if TAM inhibits CaM KI-mediated transcription," *in vitro* competition experiments were performed. TAM had previously been shown to be antagonistic to CaM function by inhibiting CaM-dependent activation of phosphodiesterase (PDE) (27). We wanted to determine if this was due to competitive binding of TAM to CaM. As an initial approach to this goal we characterized the inhibition constant for TAM inhibition of CaM activation of CaM KI. Increasing concentrations of either the CaM antagonist, W7 or TAM was preincubated in the reaction mix containing 10 nM CaM for 30 minutes at 30 °C. 1 nM CaM KI was then added to start the 10 minute kinase reaction. As shown in figure 1, even concentrations of 100 μM TAM inhibited CaM KI activity by only 20%. W7, however, inhibited >90% of the CaM KI activity at a concentration of 40 μM. CaM KI has a very low K_{CaM}, or concentration of CaM required for half maximal activity. It is likely that the affinity of CaM for TAM is much lower than the affinity of CaM for CaM KI. It is also possible that TAM can bind to CaM without inhibiting its ability to activate CaM KI. Our lab has shown that different CaM-dependent enzymes are activated by CaM in different manners (28, 29). It is possible that PDE is inhibited by TAM whereas CaM KI is not. At any rate, since TAM has very small effects on CaM KI activity, we were reluctant to perform experiments to determine if TAM inhibits CaM KI dependent transcriptional activation. Any inhibition seen would probably be due to nonspecific effects of the drug rather than of inhibition of the CaM KI pathway. Therefore, these experiments were not pursued further.

Task #1 was to "determine if CaM KI phosphorylation is required to stimulate CREB-mediated transcription in human breast cancer cells." Initially, I had a very difficult time recapitulating published experiments which demonstrated that CaM KI stimulated CRE dependent transcription. We began transfection experiments not in breast cancer cells, but in cells in which I had previously used successfully the Jurkat cell line (human T cells) and the HaCaT cell line (human keratinocytes). The induction of CRE as well as GAL4-CREB transcription in both of these cell types in response to evelated intracellular Ca^{2+} is relatively small, 6-8 fold (figure 2 A, data for HaCaT cells). We then obtained R. Maurer's GH3 (pituitary cells) as a positive control. In these cells, Ca^{2+}/CaM independent 1-295 CaM KI has been shown to stimulate GAL4-CREB transcription 50 fold (16). Indeed, in these cells, we found 1-294 CaM KI could stimulate transcription over 200 fold. I was interested in why two cell lines, both of which contain endogenous CaM KI and have the capacity to respond to Ca^{2+} mediated signaling events, would markedly differ in their transcriptional inducibility in response to CaM KI.

As a continuation of these observations, we did pCREB western blots in which an antibody specific for phosphorylated S133, the activating residue in CREB, was used. Ionomycin, a Ca^{2+} ionophore, could stimulate CRE transcription but yet did not transiently increase the levels of pCREB (figure 2 B). Indeed, CREB is constitutively phosphorylated in these cells. The positive control for this experiment was the rat PC12 cell line in which NGF is known to cause a transient increase in pCREB. CRE dependent transcription in all cells is known to require S133 and the signal dependent activation of transcription is thought to occur via this phosphorylation. Recently, however, Bading's group has suggested that one could uncouple activation of CRE transcription from phosphorylation of S133 (30, 31). We then hypothesized that perhaps ionomycin does not exert its effects on CREB, but on the CREB-binding protein (CBP). We obtained GAL4-CBP constructs from R. Goodman to determine whether we could stimulate CBP transactivation by ionomycin. The most responsive construct was CBPIII, containing amino acid residues

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451-682. Encompassed in this construct is the CREB binding domain of CBP. We found that pCREB binding to this fragment is necessary for transcriptional activation. Deletion of the last 20 amino acids or a point mutant of R600Q both of which attenuate pCREB binding *in vitro* (figure 3B) are inactive for basal transcription as well as in response to ionomycin. The viral oncoprotein, E1A, which had previously been shown to bind to two regions in the C-terminus of CBP, can also bind directly to 451-682 to inhibit transcriptional activation (data not shown) by competing with pCREB(figure 3A). We've determined that residues 662-682 are critical in several aspects. These residues are required for transcriptional activation (data not shown), pCREB binding, and E1A binding (figure 3B). Residue S670 is crucial for basal and ionomycin stimulated transcription (figure 4A). Since mutation of this residue does not affect pCREB binding (figure 3B), we propose that another factor required for basal transcriptional activity recognizes S670. A model summerizing these ideas is presented in Figure 6. Preliminary results suggest that several diferent proteins bind to CBP S670E affinity columns but not to CBP S670A affinity columns. We are currently working on a manuscript that includes the above data.

We were interested to determine how ionomycin stimulated CBPIII mediated transcription. 1-294 CaM KI along with KKB stimulates CBPIII transcription several fold (data not shown). Ca^{2+}/CaM independent CaM KIV had very little effect. 1-290 CaM KII completely blocks both basal transcriptional activation and the ionomycin stimulated induction of CBPIII (figure 4B). KN-93 treatment (Task #3 C) of the cells resulted in a two fold increase in CBPIII transcription consistent with the idea that inhibition of CaM KII *in vivo* would cause CBPIII transcription to increase (data not shown). Since KN-93 also inhibits CaM KI and IV, both of which are expressed in this cell line, ionomycin stimulation of CBPIII is not mediated by these two kinases since drug treatment increases rather than decreases transcription. It appears that ionomycin may exert it's effects not through a CaM dependent kinase but through the Ca^{2+} dependent nuclear PKC isoform, ß2.

The overall aim of Task #1 was to determine whether CaM KI activation was required for CREB mediated transcription. Indeed, we have collected data that suggest that CaM KI is activated by KKB *in vivo* which in turn results in a stimulation of CREB dependent transcription (32) and also in CBP dependent transactivation (this document). However, using a Ca^{2+}/CaM dependent CaM KI, some CREB-dependent transcription occurs in the absence of ionomycin stimulation (16, 32). These data indicate that a proportion of CaM KI is active at ambient Ca^{2+} concentrations. As it is unclear as to whether CaM KI is phosphorylated at T177 or not under resting cell conditions. I have made the T177A, 1-294 mammalian expression vector to test whether basal CaM KI stimulation of CREB mediated transcription is dependent on CaM KI phosphorylation.

The possibility that CaM KI could stimulate transcription in the absence of activation caused us to reflect on peptide substrate data that we had generated. We found that peptides derived from the yeast transcription factor ADR1 were extremely good substrates for unphosphorylated CaM KI. We have since characterized the kinetic mechanism responsible for these effects and found that the primary parameter changed upon activation of CaM KI by KK was a >40 fold reduction in the K_m for substrate. The unphosphorylated CaM KI has a K_m and V_{max} for ADR1 that are similar to those seen with activated enzyme using synapsin site 1 peptides (Table I). Likewise, when ADR1 is the substrate, phosphorylation of the activating residue, T177, or changing T177 to A have very little effect on the specific activity (Figure 5A). We have synthesized chimeric peptides betwen site 1 and ADR1 to determine which residues within ADR1 convey activation-independent substrate specificity. We found that the addition of LKK at positions P-8, P-7, and P-6 (relative to the phosphoacceptor site) onto the site 1 peptide converts site 1 into an activation independent substrate (Table I). We then made substitutions within the ADR1 sequence to ascertain whether the hydrophobic or basic residues at these positions were responsible for this effect. The ability of CaM KI to circumvent the need for activation is due to the N-terminal basic residues at P-7 and P-6 with the largest effect due to the K at P-7. We are proposing that CaM KI activation serves

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to broaden the substrate specificity and is not merely an "on-off" switch for kinase activity as was previously postulated. A manuscript relating to these experiments is currently being written and will be very relevant to how CaM KI can function in cells, particularily if different substrates require alternate modes of enzyme activation. It may be possible that one form of activation and substrate preference is favored in breast cancer cells.

In Task #1 sections C,D,and E, we proposed to determine which CaM KK are expressed in cells by western blot, determine which CaM KKs activate CaM KI *in vivo*, and determine whether the CaM KKs are regulated by extracellular signals. Our collaborator, Arthur Edelman, has generated peptide antibodies to the N and C terminus of both CaM KKA and CaM KKB. These antibodies have been affinity purified but are very poor in both western blots (1:40 dilution) and in immunohistochemistry as we have difficulty detecting bacterially expressed and purified kinase kinase on a western blot and signals from cell and tissue extracts are virtually nonexistant. We know based on the tissue purification protocol for the kinase kinases that these proteins are of very low abundance. The combination of low protein abundance as well as poor antibodies has been a great hindrance to advancing our knowledge of the profile of endogenous expression the the CaM KKs. We are currently trying to generate better antibodies so that Task #1 C can be completed during the next year.

Task #1 D is to "identify which CaM KKs activate CaM KI *in vivo*." We have demonstrated that the stimulation of both CREB and CBP dependent transcription by CaM KI can be enhanced by cotransfection of either KKB or a Ca^{2+}/CaM independent form of KKB, 1-487. We have not transfected KKA to see if it too can enhance CaM KI stimulation of transcription because at this time, we do not have a mammalian expression vector expressing KKA. Tokumitsu et al. (33) have shown, however, that CaM KI can be activated *in vitro* by lysates expressing the rat KKA. Therefore, it is very likely that KKA will also enhance CaM KI stimulation of transcription. We have not yet attempted to determine which extracellular signals activate the CaM KK pathway (Task #1 E).

Task #3 is "Identify other components of the CaM KI pathway, other than calmodulin, that can be inhibited." We do have the CaM KKA kinase minus mammalian expression vector as well as the CREB S133A vector (section A). Stable cell lines using these vectors have not yet been made (section B). Bading et al. (34) have shown that the CaM kinase inhibitor KN-62 can attenuate c-fos transcription in PC12 cells. This inhibitory activity has been mapped to the calcium response element (CaRE) which is almost identical in sequence to a CRE. Thus, it is likely that the KN compounds would also inhibit CRE mediated transcription. However, we will need to further characterize these potential pathway inhibitors to determine which would most likely cause phenotypic changes in Tasks #4 and 5.

Preliminary data relating to Tasks #4 and 5, indicate that protein levels of CaM KI may be regulated during the cell cycle. As the cells reenter the cell cycle from growth arrest, CaM KI expression dramatically peaks. It will be interesting to see at which stages during the cell cycle CaM KI is functionally important and whether these stages are compromised in our human breast cancer cell lines.

Methods

CaM KI inhibition assay

Various concentrations of W7 and TAM were preincubated for 30 minutes at 30 oC in a reaction buffer containing 50 mM hepes, pH 7.5, 5 mM mgCl$_2$, 1 mM CaCl$_2$, 1 mM ATT, 0.5 mg/ml BSA, 10 nM CaM, 0.1% tween-80, 0.4 mM ATP, 0.2 mM ADR1 substrate, and 1 μCi/reaction γ^{32}P-ATP. The 10 minute kinase assay was started by the addition of 1 nM CaM KI (previously diluted out in 50 mM hepes, pH 7.5, 0.5 mg/ml BSA, and 0.1%

tween-80. Reactions were terminated by spotting on p81 filters and quantitated as described (1).

Tranfection and Luciferase Assays

HaCaT cells were plated at a density of 175,000 cells per well in 6-well plates in α-MEM media (Gibco-BRL), 10% fetal bovine serum , (2 mM) L-Glutamine (Gibco-BRL) , (50 U/ml) Penicillin-G/ (50 μg/ml) Streptomycin Sulfate (Gibco-BRL) and grown overnight. The next day, the cells were incubated in 100 μM chloroquine containing MEM and each well transfected with a DEAE-dextran (150 μg) /DNA mix for 3 hours. The chloroquine containing media was then removed, the cells washed in PBS, and glycerol shocked for 2 min and incubated in growth media overnight. 12 hours post-shock, cells were treated with either 100 pM TGFß or 1.5 μM ionomycin, or left untreated, and harvested 20 to 24 hours later. Soluble extracts were prepared from Luciferase Lysis Buffer (300 μl/well) containing 1% Triton-X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT and 25 mM glycyl glycine (pH 7.8), and centrifuged at 14,000 rpm for 5 min at 4°C. 100 μl of lysate was mixed with 200 μl reaction mix composed of (for 300 μl vol): 25 mM glycyl glycine (pH 7.8), 18.3 mM $MgSO_4$, 6.2 mM ATP (pH 7.0), and 200 μg BSA. Luciferase activity was assayed by integrating total light emission over 30 s using a Berthold luminometer upon automatic injection of 100 μl (0.25 mM) luciferin (ICN) into the reaction mix. ß-galactosidase activity was assayed by mixing 50 μl lysis buffer with 100 μl lac-Z buffer (0.06 M Na_2HPO_4-7H2O, 0.01 M KCl, 0.039 M NaH_2PO_4-H_2O, 0.001 M MgSO4-7H2O, 0.27% ß-mercaptoethanol) containing the substrate, chlorophenolred-ß-D-galactospyranoside (Boehringer Mannheim), incubating at 37°C for approximately 3 hours and reading the optical density at 575 nm).

pCREB Western blots

HaCaT cells were plated in 10-cm culture plates at a density of $2x10^6$ cells per plate. Cells were grown overnight in culture media. The next day, cells were treated with ionomycin for 10 min, or left untreated. Pheochromocytoma (PC12) cells were also plated in 10-cm culture plates at a density of $1x10^6$ cells per plate. Each plate was pre-coated with 10% collagen (Collaborative Biomedical Products). Cells were grown overnight in DMEM (Gibco-BRL) supplemented with 10% FBS and 5% horse serum. The next day, cells were treated with NGF for 10 min or left untreated. Cells were washed in tris-buffered saline two times before addition of 150 μl boiling 2x Laemmli buffer to the 10-cm plate. Samples were ran on 12% acrylamide gels subjected to immunoblot analysis.

Generation of mutant CBPIII constructs

Mutant CBPIII constructs were made by subcloning GAL4-CBPIII into pbsk (Promega) containing an F1 origin of replication to generate pbsk-pGAL4-CBPIII and transforming into CJ236 bacteria. Addition of the kanamycin resistant helper phage VCS M13, selection with kanamycin, and overnight growth, led to the generation of single stranded DNA which was precipitated with a solution of 20% PEG-8000 and 1.5 M NaCl and phenol/chloroform purified. Mutant primers for CBPIII were generated for R600Q (5' GTGACTCAGGACCTACAGAGTCATCTAGTC 3'), 670A (5' GAAGAAAAGCGGAGGGCGCGCTTACATAAGCAAG 3'), and 670E (5' GAAGAAAAGCGGAGGGAGCGGTTACATAAGCAAG 3'). Primers were phosphorylated with T4 Polynucleotide kinase and annealed to the DNA. DNA was synthesized by addition of 1x synthesis buffer, 2.5 mM dNTPs, T4 DNA ligase and T4 DNA Polymerase and incubated at 37°C for 45 min. The reaction was terminated by

freezing, and transformed into the bacterial strain JM83 where only the CBPIII mutant strand survived to replicate its DNA. After generation of mutant pbsk-pGAL4-CBPIII, the mutant CBPIII was spliced out using BamHI sites and subcloned in frame into pGAL4 (R600Q, S670A, S670E) and pGEX-3X (R600Q, S670A S670E). CBPIII 451-661 was generated by PCR from pGEX-CBPIII 451-682 with an engineered stop site and BamHI site near the C-terminus of CBPIII (5' GAAAATCTATAAAATACAATAGGGATCCGCGTGC 3'). The PCR product was subsequently subcloned into pGEM-T (Promega) and spliced out using BamHI. This shortened mutant (451-661), was ligated to pGAL4 and pGEX-3X to determine both transactivation potential and binding function.

Expression and purification of GST-CBPIII

Bacterial strain DH5α containing GST-CBPIII and CBPIII mutants were grown in 5 ml Luria Broth (LB) media overnight at 37°C. The next day, the cultures were transferred to flasks containing 50 ml of LB and shaken vigorously for one hour (OD approximately 0.6) at 37°C. 0.5 mM IPTG was then added to the culture and shaken vigorously for another 4 hours at 37°C. The bacteria was then spun down at 3000 rpm for 10 min. The pellet was resuspended in lysis buffer (MTPBS⁻) containing 1% Triton X-100, 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 pH 7.3, 1 mM PMSF, 1 mM DTT, protease inhibitors, 1mM $NaVO_3$, and 20 mM NaF. Cells were sonicated 4 times on ice in 30 s intervals. Lysates were clarified by centrifugation at 7000 rpm before addition of 500 µl of a 50% slurry of lysis-buffer equilibrated glutathione beads. After a 4 hours incubation at 4°C, the beads were pelleted by centrifugation at 1000 rpm and washed 3 times in lysis buffer before resuspension in 400 µl lysis buffer.

E1A binding assay

HaCaT cells were plated in 10-cm culture plates at a density of $2x10^6$ cells per plate. Cells were then incubated at 37°C in 3 ml of serum-free medium with a multiplicity of infection of 1000 of recombinant adenovirus containing 12S E1A or 12S E1A RG2 for 4 hours. 5 ml of culture medium containing 10% FBS was then added and the cells incubated for an additional 20 hours. Soluble lysates were generated in Universal Lysis Buffer (ULB) containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 0.2 mM sodium molybdate, 20 mM ß-glycerophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors.
Approximately 200 µg of E1A containing lysates were incubated with Glutathione-Sepharose bound GST-CBPIII or GST-CBPIII mutants (10 µg) for 12 hours at 4°C with constant rotating. The beads were collected and washed 3 times (3 min per wash) with ULB. Bound proteins were eluted by boiling in 1x Laemmli sample buffer and subjected to immunoblot analysis.

CREB binding assay

Bacterially expressed and purified CREB (250 ng/rxn) (gift of S. Shenolikar) was phosphorylated by PKA for 20 min @ 30°C in the presence of 50 mM tris pH 7.5, 10 mM $MgCl_2$, 500 µM ATP, 1 mM DTT, and (2 µCi/rxn) $\gamma^{32}P$-ATP (Amersham). Reactions were terminated by the addition of 7.5 µM PKI. 4 µg GST or GST-CBPIII plus or minus increasing concentrations of 6xHis-E1A were added to the pCREB. The volume of each reaction was 600 µl in ULB buffer with a final PKI concentration of 0.5 µM. PKI was added to prevent PKA phosphorylation of E1A (36). Tubes were rotated at 4°C for 12

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hours and washed 3 times with 1 ml of ULB. Samples were pelleted, solubilized in 30 µl ULB with 10x Sample buffer and run on a 12% acrylamide gel and visualized by autoradiography.

Kinetic Analysis

The five to six ATP concentrations ranged from 400 µM to 5 mM, while the five peptide concentrations ranged from 100 µM to 1 mM for ADR varients and from 200 µM to 1.2 mM for site 1 varients. 1 ng unactivated CaM KI was assayed for 15 minutes using site 1 and for 10 minutes using ADR1. 1 ng activated CaM KI was assayed for 5 minutes with all peptide substrates. The kinase buffer is as described in figure 1.

Figure 1
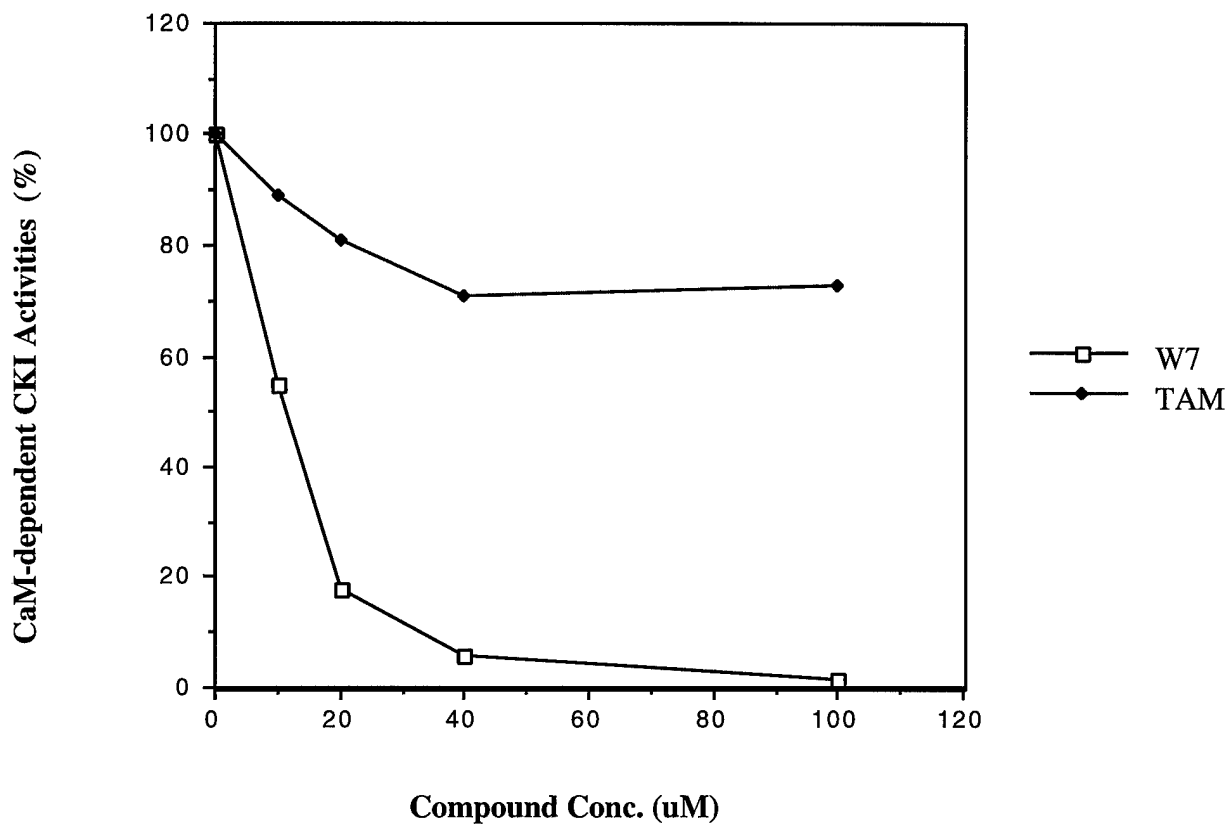
Figure 1. CaM-Dependent activity of CaM KI with increasing concentrations of the CaM inhibitor, W7 and the anti-estrogen, TAM.
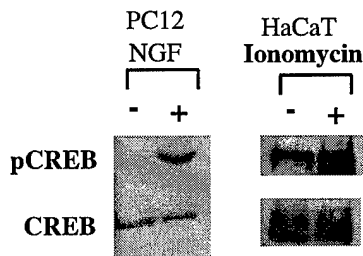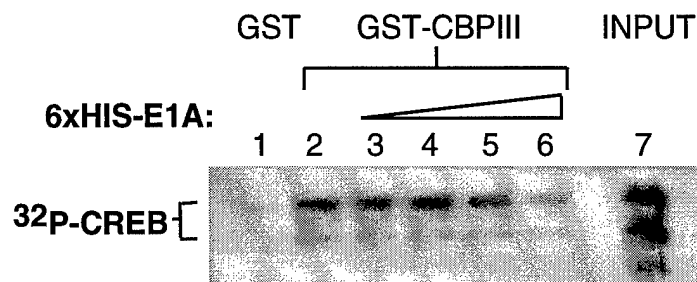
Figure 2 A
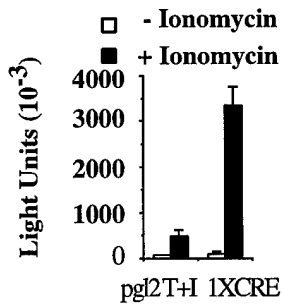
B
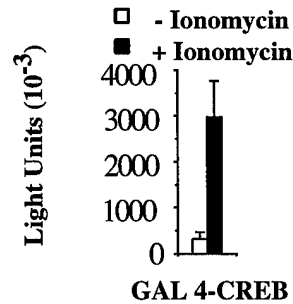
C

Figure 2. Ionomycin stimulates CRE-dependent transcription but does not increase phosphorylation of Ser-133 on CREB. (A) Ionomycin stimulates CRE-mediated transcription. A luciferase reporter plasmid (pgl2 T + I) containing only a TATA box (T) and initiator sequence (I) was attached to a consensus CRE (TGACGTCA), transiently transfected, and treated with 1.5 µM ionomycin 24 hours prior to harvesting. Transfection efficiency was calibrated by co-transfection with pCMV-ßgal. Error bars denote standard deviation of two separate transfections performed in the same experimental set. (B) Ionomycin induces CREB-dependent transcription. A luciferase reporter plasmid containing only a TATA box and initiator sequence attached to five concatemerized Gal4 sites (5xGal4 T+I) and a Gal4-CREB fusion plasmid were co-transfected and the cells were treated with 1.5 µM ionomycin 20 to 24 hours prior to harvesting. (C). CREB is constitutively phosphorylated on Ser-133 in HaCaT cells. Ionomycin treatment does not lead to an increase in pCREB. A 10 min treatment with ionomycin does not affect the level of pCREB whereas a 10 min NGF-treatment of PC12 cells leads to a dramatic increase in phosphorylation of Ser-133 on CREB. Treated cells were lysed in boiling Laemmli sample buffer and subjected to immunoblot analysis. The pCREB blot was performed using pCREB specific antibody that was the generous gift of D. Ginty. a-CREB antibody (Upstate Biotechnology Inc.) recognizes both phosphorylated and unphosphorylated CREB.
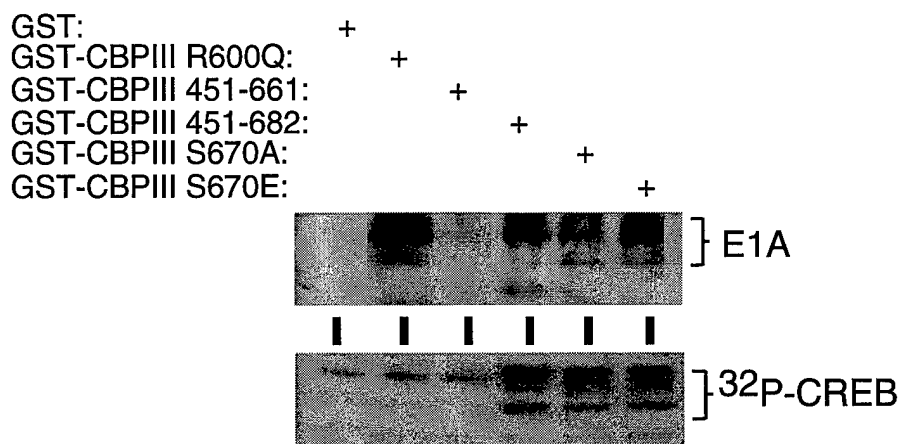
14

Figure 3 A

Figure 3. E1A binds to CBPIII and competes with CREB for CBPIII binding. (A) E1A competes with CREB for CBPIII binding. CREB was labeled with γ32P-ATP by PKA. pCREB was incubated with either GST or GST-CBPIII (lane 1, 2). Increasing concentrations of 6xHis-E1A were added to determine if E1A competes for CREB binding to CBPIII. Pulldowns were washed extensively and visualized by SDS-PAGE and autoradiography. Input denotes 50% of the 32P-CREB used in the pulldown assay. (B) Analysis of CBPIII mutants for CREB and E1A binding. Using site-directed mutagenesis, amino acid 670 in CBPIII was changed to an Ala or a Glu, and attached in frame to GST to create GST-CBPIII S670A and GST-CBPIII S670E, respectively. In the top panel, glutathione-bound GST-CBPIII and its mutants (10 μg) were incubated with lysate (200 μg) from adenoviral-12S E1A-infected HaCaT and an E1A western blot performed. In the lower panel, CREB was labeled with PKA. Binding reactions were performed with GST-CBPIII and its mutants. After extensive washes in Universal Lysis Buffer, the reactions were resolved on SDS-PAGE and visualized with autoradiography.
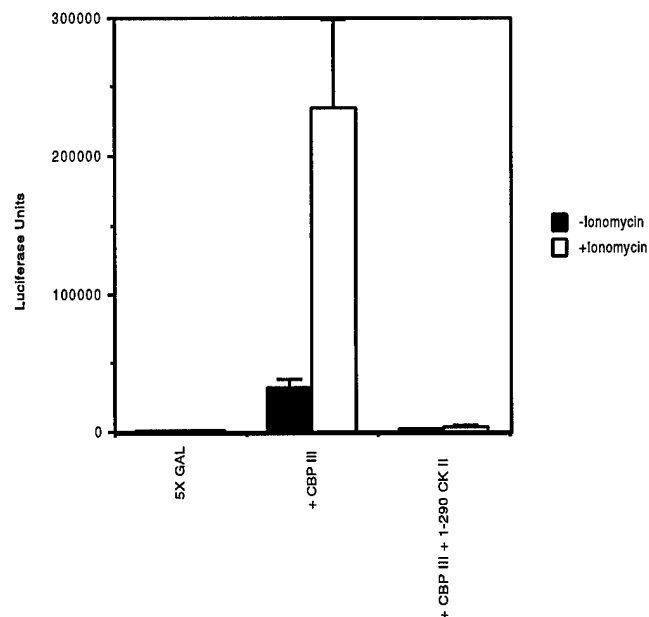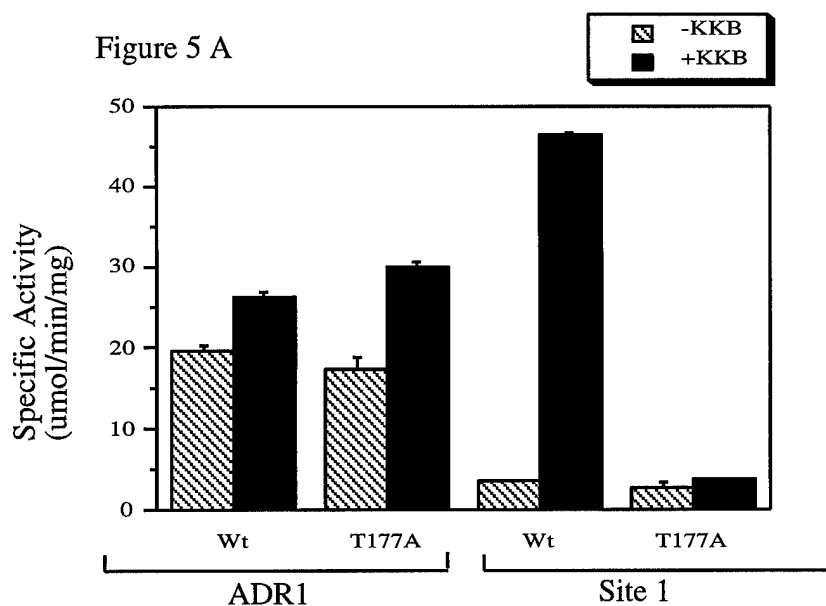
Figure 4 A
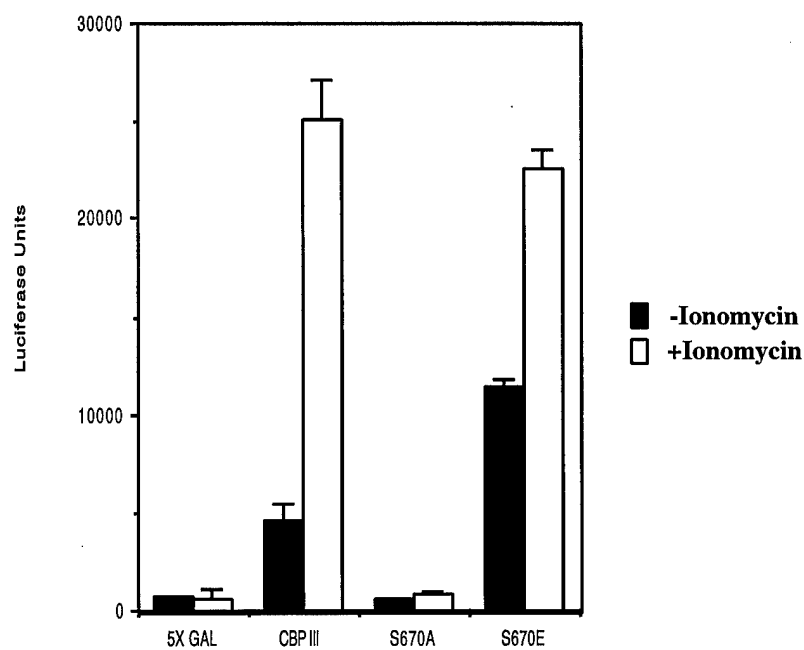
B

Figure 4. (A) Ser-670 of CBPIII is necessary for ionomycin stimulation of CBPIII-dependent transcription. Wild-type GAL4-CBPIII as well as the S670A or S670E mutants was transiently co-transfected with the 5XGAL4 T+I reporter and stimulated with ionomycin for 24 hours. Cell lysates were made and luciferase and ß-galactosidase activity assayed. (B) 1-290 CaM KII blocks ionomycin stimulation of CBPIII-dependent transcription. Wild-type GAL4-CBPIII was transiently transfected with or without 1-290 CaM KII and stimulated with ionomycin for 24 hours prior to harvesting.

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Table I

Unactivated				Activated		
	Km (uM)	Vmax (umol/ min/mg)	Vmax/ Km	Km	Vmax	Vmax/ Km
Site 1 LRRRLSDANF	209	26.1	0.13	4.7	33.1	7.04
ADR1 **LKKLTRRASFSGQ**	17.4	39.4	1.98	7.1	87.0	12.25
LKKLRRRLSDANF	6.7	40.4	6.03	2.89	63.33	21.91

Kinetic analysis of site 1, ADR1, and chimeric peptides. Assay conditions were as described in experimental methods. The Km for ATP decreases about 2 fold upon CaM KI activation using either the site 1 peptide or ADR1 (110 μM to 61 μM.) The results shown in the table represent an average of four experiments for unactivated CaM KI and one experiment for activated CaM KI.
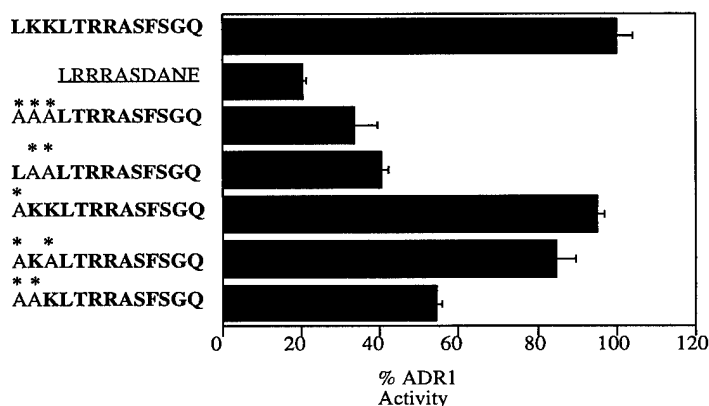
Figure 5 A

B

Figure 5 A. Specific activities of unactivated and activated CaM KI with site 1 and ADR1 substrates. CaM KI (10 ng) was preincubated with 563 ng KKB for 20 minutes, then assayed as described in experimental procedures for 3 minutes. CaM KI without KKB was not pre-incubated alone because the enzyme lost activity during the pre-incubation. Instead, the kinase was assayed for 3 minutes without prior pre-incubation. Final concentration of peptide was 200 µM and ATP was 500 µM. The results here are a single representative experiment. B. Kinase assays using ADR1 peptides with substitutions at P-8 through P-6. ADR1 sequences are shown in bold. Site 1 sequences are underlined. Changes within the ADR1 sequence at P-8, P-7, and P-6 are shown with an asterisk. Assay conditions are as described infigure 2B. The results shown here is a single experiment in triplicate.
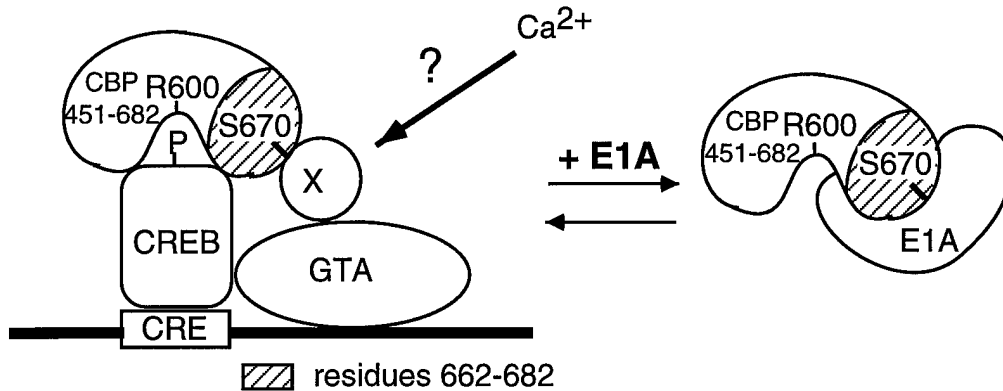
18

Figure 6

Figure 6. Model for pCREB-dependent CBPIII(451-682) transcriptional activation in response to Ca^{2+}. R600 and residues 662-682 (the hatched region of CBP) are required for transcriptional activation and also for pCREB binding. Since pCREB interaction is required even in the absence of CREB DNA binding, as with the GAL4-CBPIII(451-682) fusion protein, we presume that pCREB is required to make additional contacts with components of the general transcriptional apparatus (GTA) such as those proposed with TAFII 130. E1A competes for pCREB binding to CBPIII in a manner that requires residues 662-682 but is independent of R600 or S670. S670 is critical for both basal transcription and stimulation by either Ca^{2+}. Since mutations of S670 do not affect pCREB binding, we propose that this residue is critical for binding some factor, X, that is required for transcription. It seems possible that X could be the target of the signaling cascades initiated by Ca^{2+}. Whether X is a novel protein or a known component of the GTA remains to be determined.

Conclusions

Through these studies, we have made findings that identify further complexity to the stimulation of CRE transcription (figure 6). CaM KI acts primarily by phosphorylation of S133 of CREB to promote either CRE or GAL4-CREB dependent transcription. This CREB phosphorylation is required for interaction with CBP. GAL4-CBPIII 451-682 constructs are very active in stimulating basal transcription and in response to ionomycin. This transcription is dependent on pCREB binding in that 451-661 CBP and R600Q CBP have very little basal activity and are not stimulated by ionomycin. Suprisingly, the oncoprotein E1A can bind directly to 451-682 CBP and inhibit its function by competing with pCREB. Residues 661-682 are critical for pCREB binding, E1A binding, and transcriptional activity. Residue S670 within this 21 amino acid region is required for all transcription. Since S670A does not abrogate pCREB binding, we propose this residue may be a docking site for another component of the general transcriptional apparatus.

We have also determined that the biochemical mechanism of CaM KI activation is primarily through a 40 fold reduction in K_m for substrate (site 1 K_m decreases from 209 μM to 4.7 μM). The ADR1 peptide is a very good substrate for unactivated CaM KI because it has a K_m of 17 μM. Activation of CaM KI, has only modest effects on the K_m and V_{max} using ADR1, indicating CaM KI activity is activation independent. Peptide mutational studies indicate that the most important residue for this effect is K at P-7. We propose that activation loop phosphorylation changes the substrate specificity of CaM KI. Since many ser/thr, tyr, and receptor kinases have been shown to be regulated by activation loop phosphorylation, it is possible that activation independent substrates may also exist for these kinases.

In reviewing the statement of work, we have not proven whether part of TAM's actions may be through the inhibition of the CaM kinase cascade although TAM is only a poor competitive inhibitor of CaM KI *in vitro*. In addition, we have discovered that even in cell lines where CREB is constitutively phosphorylated and is not inducible, we can still stimulate CRE dependent transcription with a Ca^{2+} signal. We must now consider this additional mechanism of regulation in analyzing our breast cancer cell lines. The inhibition of the CaM kinase cascade may cause cell cycle arrest or apoptosis and could be exploited as breast cancer therapy. Those experiments remain to be executed in the next grant year.

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